CHANGES IN ALKALINE PHOSPHATASE LEVELS IN GINGIVAL CREVICULAR FLUID AND SALIVA FOLLOWING EN-MASSE RETRACTION: A COMPARITIVE STUDY

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CERTIFICATE

This is to certify that this dissertation titled "**CHANGES IN ALKALINE PHOSPHATASE LEVELS IN GINGIVAL CREVICULAR FLUID AND SALIVA FOLLOWING EN-MASSE RETRACTION: A COMPARITIVE STUDY"** is a bonafide work done by **Dr. SHIREEN COX** under my guidance during her post graduate study period between 2012-2015.

This dissertation is submitted to **THE TAMIL NADU Dr. M.G.R. MEDICAL UNIVERSITY** in partial fulfilment for the degree of Masters in Dental Surgery, in Branch V- Orthodontics and Dentofacial Orthopaedics. It has not been submitted either partially or fully for the award of any other degree or diploma.

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CONTENTS

INTRODUCTION

Orthodontics involves the use of mechanical load to bring about tooth movement in the desired direction. The ability to move teeth inside the alveolar bone was known for over a millennia but the mechanism by which this happened remained unknown. **Farrar**, in 1888 tried to explain why teeth moved when subjected to mechanical forces. According to him, teeth move because the mechanical force bends the alveolar bone or they resorb the alveolar bone[1]. From then on *in-vitro* & *in-vivo* studies were carried out using various investigative tools to understand the cellular and tissue level changes taking place during tooth movement. The efforts of such studies brought about a logical conclusion that teeth can be moved because the cells around their roots are enticed by the orthodontic force to remodel the tissues around them.

Orthodontic tooth movement is a highly sophisticated process. It comprises a series of networked reactions in converting the mechanical force into biological signals (mechano-transduction) and orthodontic tooth movement. It is characterized by both modeling and remodeling changes in the periodontal ligament and alveolar bone, which on exposure to varying degrees of magnitude, frequency and duration of force exhibit extensive molecular level changes[2]. A thorough knowledge about these cellular and molecular level reactions that enable the bone to adapt to changes in its mechanical environment is very essential for the practice of clinical orthodontics.

In the initial days of trying to understand the biological mechanism of orthodontic tooth movement, only histological studies were carried about. The first histological examination was done by **Sandstedt**. He subjected the tooth of a dog to orthodontic force and found that tooth movement occurred by bone formation on the tension side and bone resorption on the pressure side[3]. **Oppenheim** in 1912 studied

tooth movement in a pre-adolescent baboon and found that there was complete transformation of the entire alveolar bone and suggested that the effects of orthodontic force spread beyond the limits of PDL[4].

Later, histochemical studies were carried about in order to elucidate the various enzymes and molecules participating in orthodontic tooth movement. In 1983, **Lilja *et al*** reported on the detection of various enzymes in mechanically strained paradental tissues of rodents, including acid and alkaline phosphatases, beta galactosidase, aryl transferrase & prostaglandin synthetase[5]. **Davidovitch *et al*** used immunohistochemistry methods to identify a variety of first and second messengers in cats' mechanically stressed paradental tissues. These molecules include cyclic nucleotides, prostaglandins (PGs), neurotransmitters (NTs), cytokines and growth factors (GFs)[6,7].

Gingival Crevicular Fluid (GCF) has proved to be a non-invasive and effective medium to detect the changes in these various enzymes and molecules that play an active role in bringing about orthodontic tooth movement[8]. GCF is an osmotically mediated inflammatory exudate found in the gingival sulcus[9]. Though serum is the main source of its constituents, the composition of GCF is also modified by the changes taking place in the periodontal tissues. A cascade of changes occurs in the periodontal ligament tissues during orthodontic tooth movement and there is expression of an array of biochemical factors in the GCF which feature as Biomarkers of tooth movement. These help in analyzing the biological processes that are taking place at the molecular level during orthodontic tooth movement[10].

Fabrizia d' Apuzzo[10] classifies these Biomarkers of tooth movement as:

- Biomarkers of inflammation
- Biomarkers of cell death
- Biomarkers of bone resorption
- Biomarkers of bone formation

In recent years the research trend, in dentistry and other fields of medicine, is shifting towards the use of Saliva as a diagnostic tool for the detection of various Biomarkers of systemic and oral diseases[11]. The advantages of using saliva as a detection medium for biomarkers over other body fluids like serum or GCF is its ability to be collected in an easy, non-invasive manner, at low cost and in sufficient quantities for analysis[12]. In the field of dentistry, Saliva is used as a diagnostic tool for the detection of Oral Cancers, Periodontitis, various Syndromes, Autoimmune diseases and oral mucosal pathologies[13,14,15].

In the field of Orthodontics, only a few studies have tried focusing the promising aspect of Saliva as a valuable tool in detecting the Biomarkers of orthodontic tooth movement[16,17]. In the interest of exploring more on the use of saliva as a diagnostic tool, in our study we have decided to analyze the enzyme, Alkaline Phosphatase, a Biomarker of alveolar bone formation, in saliva and GCF.

Alkaline Phosphatase is an enzyme found in the plasma membrane of osteoblasts. Since orthodontic force involves proliferation and differentiation of the PDL cells into osteoblasts and because of the important role of Alkaline Phosphatase in the mineralization of newly formed bone, they are extensively used as a bone formation biomarker[18].

In orthodontic literature, few studies have been done to study the Alkaline Phosphatase activity during orthodontic tooth movement, both in rats and humans. **Stephen Keeling *et al* (1993)** studied Alkaline Phosphatase and Acid Phosphatase changes in serum and alveolar bone during orthodontic tooth movement in rats. This study supports the finding that bone remodeling is characterized by tandem periods of activation, resorption, reversal and formation and also found high correlation of these stages with the enzymes mentioned above[19]. It was **Michael Insoft *et al* (1996)** who first did a human study on Alkaline Phosphatase activity in GCF during orthodontic tooth movement[20].

Saliva as a tool to study the role of Alkaline Phosphatase in orthodontic tooth movement has not been studied so far in the orthodontic literature. The previous studies done on human GCF analyzed the activity of Alkaline Phosphatase only during single tooth movements such as canine retraction and molar distalization[21,22]. Activity of the enzyme in complex tooth movements like en-mass retraction, during which both modeling and remodeling changes happen in bone, has not been reported.

Hence in our study we have focused on studying the activity of the enzyme, Alkaline Phosphatase during en-masse retraction with a continuous force of 150 gram using NiTinol coil spring and compare its activity in both Gingival Crevicular Fluid and Saliva.

AIMS&OBJECTIVES

1. To quantitatively estimate and compare the levels of the enzyme, Alkaline Phosphatase in Gingival Crevicular Fluid before and during the application of a continuous force for en-masse retraction at various time intervals.
2. To quantitatively estimate and compare the levels of the enzyme, Alkaline Phosphatase in Saliva before and during the application of a continuous force for en-masse retraction at various time intervals.
3. To compare the pattern of rise of the enzyme, Alkaline Phosphatase in both Gingival Crevicular Fluid and Saliva at various time intervals during en masse retraction.
4. To explore the possibility of using Saliva as a diagnostic medium for reliable detection of the enzyme Alkaline Phosphatase in orthodontic clinical practice.

REVIEW OF LITERATURE

Oppenheim[4] (1912) reported tooth movement in one pre-adolescent baboon resulted in complete transformation (remodeling) of the entire alveolar process, indicating that orthodontic force effects spread beyond the limits of the PDL

Storey and Smith[23] (1952) studied orthodontic force values and found that there is an optimum range of force that produces a maximum rate of distal movement of canine and that this optimum force did not produce discernible movement of the molar anchor unit in first premolar extraction cases. They also found that the original force range for moving the canine distally extended from 150 to 250gms.

Reitan[24] (1957) stated that an optimal orthodontic force moves teeth efficiently into their desired position, without causing discomfort or tissue damage to the patient. To achieve ideal tissue and cellular response to orthodontic loads they favored the use of light intermittent forces.

Egelberg[25] (1966) showed that the production of gingival crevicular fluid is essentially related to an inflammatory increase in permeability of the vessels underlying the sulcular and junctional epithelium.

Burstone[26] (1959) demonstrated high acid phosphatase activities in resorbing cells such as osteoclast and macrophages.

Skidmore[27] (1960) studied the nature of alkaline phosphatase enzyme in saliva. He found that there is no predetermined normal activity of ALP in saliva as it is influenced to such an extent by internal and external factors. He also found that

collected saliva can be sealed and refrigerated for 30 minutes before testing without any decrease in enzyme activity. And saliva that is sealed and frozen can be stored for a maximum period of 6 weeks without significant decrease in enzyme activity. The frozen saliva should be allowed to thaw at room temperature before testing.

Burstone[28] (1962) divided tissue reaction during displacement of teeth into three phases:

- An initial phase- is characterized by a period of very rapid tooth movement due to displacement of tooth in PDL and normally lasts a few days.
- The Lag phase- which usually lasts from one to three weeks during which the tooth does not move or has a relatively low rate of displacement. This is due to hyalinization of PDL in areas of maximum stress.
- The Post Lag phase- is when the rate of tooth movement gradually or suddenly increases after removal of hyaline zone.

Baumrind[29] (1969) proposed that the PDL is a continuous hydrostatic system and any force delivered to it will be transmitted equally to all regions of the PDL. He considers PDL as a 'Viscoelastic System'. On force application, all three structures, tooth, PDL, alveolar bone, are deformed and the amount of deformation is determined by the elastic properties of each tissue component. Based on this, he put forth the 'Bone Bending Theory'. When an orthodontic appliance is activated, forces delivered to the tooth are transmitted to all tissues near force application. These forces bend bone and following bone bending, bone turn over and renewal of cellular and inorganic fractions occur.

Hermanson[30] (1972) quantitatively determined the amount and extent of bone formation in cats incident to orthodontic tooth movement over a thirty three day period. Light continuous forces were used and the forces increased toward the end of the experiment. He found that at the tension side, bone formation peaked at 6, 15, 27 and 33 days.

Heller et al.[31] (1979) studied effect of metabolic alteration of periodontal fibres on orthodontic tooth movement by applying orthodontic force to molars of rats treated with the lathyrogen beta- aminopropionitrile. The result of this study infers that fiber tension on the alveolus may not be absolutely necessary to stimulate bone formation. Distortion of the alveolus related to force application may be a more important factor initiating bone response.

Assar Ronnerman[32] (1980) studied the reactions of gingival tissue to orthodontic closure of extraction sites. In the tissue specimen near bone tissue there was a strong alkaline phosphatase activity and comparatively low acid phosphatase activity, indicating active bone formation rather than resorption.

Robert Rej and Jean Pierre Breataudiere[33] (1980) studied on the interaction of metal ions with Alkaline Phosphatase activity. They found that ions of Beryllium, Iron, Manganese, Cobalt, Nickel, Chromium, Cadmium, Aluminium and Tin had an inhibitory effect on Alkaline Phophatase activity. Zinc ion had a stimulatory effect and Magnesium ion promoted the stimulation or inhibition of other ions.

Midgett et al.[34] (1981) reported effect of altered bone metabolism on orthodontic tooth movement by studying how bone remodeling changes induced by nutritional hyperparathyroidism affect tooth movement through alveolar bone. They showed that, in addition to applied force, tooth movement is dependent upon the state of calcium metabolism in alveolar bone.

G. Cimasoni[9] (1983) has defined Gingial Crevicular Fluid (GCF) as an exudate that can be harvested from the gingival sulcus or periodontal pocket using filter paper strips, capillary micropipettes or gingival washings.

Lilja et al.[5] (1983) reported cellular enzyme level changes associated with tissue degradation following orthodontic tooth movement. The results indicated that macrophages in various stages of differentiation were responsible for the degradation of the hyaline zone and alveolar bone during orthodontic tooth movement. They detected various enzymes in mechanically strained paradental tissues of rodents, including acid and alkaline phosphatases, beta galactosidase, aryltransferrase & prostaglandin synthetase

Davidovitch et al.[6] (1984) found that local injections of PGE and minute electric currents applied locally caused fluctuations in cyclic nucleotide and prostaglandin cellular levels producing significant increase in rate of tooth movement.

Lilja et al[35] (1984) studied the activity of alkaline phosphatase and the incorporation of tetracycline as signs of bone formation after orthodontic tooth movement for 10 hours to 6 days in rats. Defined high and low forces were used. Orthodontic forces gradually inhibited alkaline phosphatase mainly vandate and levamisole resistant ones and tetracycline incorporation on the bone surfaces in the pressure zones in the PDM depending on the magnitude of the force. It was also suggested that the disappearance of these iso-enzymes into a limited area as seen in the pressure zones was associated with inhibited bone formation and subsequent initiation of bone resorption. On tension side a slight reduction and redistribution of vandate and levamisole resistant alkaline phosphatase activity could be noted irrespective of the magnitude of the applied force.

Yamasaki et al[36](1984) found that injection of biochemical agents such as PG has been an effective method that significantly increases orthodontic tooth movement. The mechanism of action of PGE2 can be explained by the pressure-tension theory which assumes chemical signals to be stimulants that lead to tooth movement.

Daniel et al[37] (1986) discussed the newer method of quantitatively determining the active disease sites in perodontitis with response to therapy. Analysis of total alkaline phosphatase on GCF was said to increase and reflect local tissue changes and acid phosphatase was not consistent with the disease activity in periodontal disease.

Garner et al[38] (1986) studied a combination of Nitinol, beta titanium and stainless steel arch wires as to force required to overcome a simulated canine retraction assembly. Results showed a significantly larger force required during canine retraction using beta titanium followed by nitinol wires and least stainless steel wires.

Binder T.A[39] (1987) studied on acid and alkaline phosphatase levels in gingival crevicular fluid and evaluated their use as a possible indicator of periodontal disease progression. A series of timed gingival fluid samples were taken from several sites in one subject's mouth. Reproducible differences in volume of phosphatase enzyme concentrations were found between the first and subsequent samples.

Davidovitch et al.[7] (1988) tested the hypothesis that tissue remodeling during orthodontic tooth movement is modulated atleast in part by factors derived from nervous and vascular systems specifically the neurotransmitter, SP and cytokines, IL-1 alpha and IL-1 beta. Increased staining of these agents was found in areas of PDL tension and compression zones at different time periods. The results support the hypothesis that neurotransmitters and cytokines play a regulatory role in orthodontic force induced alveolar bone remodeling.

Wenchen Lee[40] (1990) studied the effect of Prostaglandin E, administered locally and systemically, to rat to study the difference in efficacy of two method of administrating in accelearating bone resorption. The results showed that there was a marked increase in number of osteoclast and Howship's lacunae in treatment groups than that of controls. Also, the systemic administration of PGE had a more marked effect on bone resorption than the local administration.

King et al[41] (1991) conducted histomorphometric study on alveolar bone turn over during orthodontic tooth movement in animal models. They demonstrated that during tooth movement, an early wave of resorption (3-5 days),followed by its reversal (5-7 days) and late wave of bone deposition (7-14 days) takes place in both the pressure and tension site of the alveolar wall.

Jonathan Sandy et al[42] (1993) Osteoblasts are now recognized as the cells that control both the resorptive and the formative phases of the remodeling cycle, and receptor studies have shown them to be the target cells for resorptive agents in bone. The osteoblast is perceived as a pivotal cell, controlling many of the responses of bone to stimulation with hormones and mechanical forces. Changes in cell shape produce a range of effects mediated by membrane integral proteins (integrins) and the cytoskeleton, which may be important intransducing mechanical deformation into a meaningful biologic response.

Stephen Keeling et al.[19] (1993) examined tartrate-resistant acid phosphatase (TRAP) and alkaline phosphatase changes in serum and alveolar bone during orthodontic tooth movement cycle in rats. The effect of differing initial force magnitudes on phosphatase changes was also examined. A peak in serum acid phosphatase occurred at day 1 and in bone at day 3. A peak in serum and bone alkaline phosphatase occurred at day 7 with a significant drop at day 10 (the latter drop in contrast to elevated osteoblast numbers). He suggested differing force magnitudes may alter the timing of these bone turnover events.

Gregory King et al[43] (1994) studied alveolar bone turn over with appliance decay in rats. He found that even with 93% appliance decay, tooth movement continued which was confirmed by seeing the peak rise and fall of the enzymes, Alkaline Phosphatase, Acid Phosphatase and Osteocalcin. A peak in bone formation activity was seen around appliance decay and there was increase in Acid phosphatase and Osteocalcin after appliance decay but Alkaline Phosphatase decreased. After 93% appliance decay a second phase of bone remodeling starts with abrupt inhibition of bone formation and initiation of bone resorption.

Orban et al[44] (1994) suggested that abrupt changes in oxygen availability within the periodontium have a regulatory role in alveolar bone remodeling during orthodontic tooth movement, similar to that seen in bone growth or fracture healing. Results showed that in hypoxia cellular proliferation increased whereas alkaline phosphatase activity and collagen synthesis decreased. In contrast, in hyperoxic condition, cellular proliferation is suppressed with concomitant collagen synthesis.

Michael Insoft, King and Keeling[20] (1996) examined acid phosphatase and alkaline phosphatase in the gingival crevicular fluid to learn whether bone turnover dynamics can be monitored in human subjects during orthodontic tooth movement. Three female subjects were observed longitudinally to assess tooth movement, plaque and inflammation. For each subject, one randomly selected premolar served as the control and was not treated, and another was moved bucally with 100 gms of force. Alkaline phosphatase peaked between the first and third weeks, followed by an increase in acid phosphatase between the third and sixth weeks. After the first week, tooth movement

averaged 0.9mm. An additional 0.9mm of movement occurred during the next 3 weeks, followed by 1.4mm during weeks 4 to 6.

Thirty additional patients, randomly divided into headgear/biteplate, bionator and control groups were also sampled cross-sectionally at the maxillary first molars. It was found that acid phosphatase was consistently higher on the mesial than on the distal in the treatment groups. Alternating peaks of acid and alkaline phosphatase were found in GCF of treated teeth as functions of treatment duration.

King et al[45] (1997) studied alveolar bone turnover and amount of tooth movement in 144 male rats. Alkaline phosphatase values elevated in treated groups at days 5, 7 and 10. This pattern reversed at day 14. There was early elevation in osteoclasts number on the mesial and osteoblasts number on the distal that returned to control by 3 to 5 days.

P.A. Hill[46] (1998) in his review describes the four phases of bone remodelling cycle as activation, resorption, reversal and formation. He states that in humans the osteoid begins to mineralize after 13 days and it takes 124-168 days.

Samuels et al[47] (1998) studied the clinical rate of space closure between Nickel Titanium closed coil spring generating forces of 100 gm, 150 gm, 200 gm and an elastic module with a starting force of 400 gm declining to zero between visits. He concluded that maximum rate of space closure was obtained with springs generating 150 gm and 200 gm force.

Sappho Tzannetou et al[48] (1998) examined interleukin-1 beta and beta-glucuronidase in GCF in children undergoing rapid palatal expansion and found that orthopedic forces evoke changes in the levels of the inflammatory mediators.

Gao et al[49] (1999) found that alkaline phosphatase activity was highest in osteoblasts, moderate in periodontal ligament fibroblast and minimal in gingival fibroblasts. No ALP activity was found in cementoblasts.

Birte Melsen[50] (1999) histomorphometrically studied alveolar bone reaction in monkeys when force levels of 100cN, 200cN and 300cN were applied to translate premolars and molars over a period of 11 weeks. She found that at lower strain levels direct resorption and bone remodeling occurs whereas at higher strain levels bone modeling is initiated and undermining resorption occurs. She also concluded that both bone resorption and formation were influenced by change in the stress/strain distribution produced by the applied force system.

Nelson B Watts[51] (1999) discussed on the clinical utility of markers of bone remodeling. Bone remodeling is a coupled reaction of bone resorption and formation. The bone resorption markers are Tartarate Resistant Acid Phosphatase (TRAP), hydroxyproline, glutamic acid and cross linked telopeptide of type I collagen. Bone formation markers are total Alkaline phosphatase, bone specific ALP, osteocalcin and procollagen.

Carlalberta Verna *et al*[52] (2000) showed that bone turnover significantly affected rate and type of tooth movement in rats. The rate of tooth movement was increased in high bone turnover induced rats and the rate of tooth movement was decreased in low bone turn over induced rats. Also the center of rotation was altered when the bone turnover rates were different. He concluded that patient's bone metabolism can also influence the rate & type of tooth movement and should be kept in mind when treating patients with metabolic bone diseases.

In his study, he cites the reason for choosing the period of study as 21 days in most bone turnover studies. The remodeling cycle in rats is usually 21 days at 6 months of age when it attains full maturation.

Kotaro Miyoshi *et al*[53] (2001) investigated the response of periodontal tissue to orthodontic force during different times of the day in rats. He found that the formation of new bone in the whole day force application group and force application in the light hours group was twice greater than that group which received force during dark hours. This shows that both bone formation and bone resorption are active in the environmental light period than dark period.

Lindsay Hoffman[12] (2001) explains the advantages and disadvantages of saliva as an analytical medium, the collection methods and its validity in assays.

Wellington J Rody[54] (2001) quantified osteoclast recruitment at compression sites as a function of time following orthodontic force application in rats.. A significant number of BrdU positive preosteoclasts were observed in the periodontal ligament and bone surface at the day 3. The number of osteoclastic cells in the bone marrow also peaked at day 3; however the highest percentage of cells in this location was observed at day 1.

Burke et al[55] (2002) studied total secretory proteins and a cyclic adenosine monophosphate (AMP)-dependent protein kinase subunit (RII) as measured in saliva and gingival crevicular fluid (GCF) after the placement of orthodontic separators to determine if mechanical force applied to teeth affects protein secretion.

Dixon *et* al[56] (2002) compared three methods of space closure in friction mechanics using active ligatures, elastomeric chains and NiTi coil springs and concluded that NiTi springs gave the most rapid and consistent rate of space closure. He recommends the use of springs delivering 150 gram or 200 gram force level and not to stretch them more than 9mm.

Kohon et al[57] (2002) studied the changes in the periodontium and rate of tooth movement under light orthodontic forces in rats. He concluded that when light forces are used there is no lag phase as suggested by Burstone and that only two phases were seen: an initial shifting of tooth in PDL followed by a smoother rate of movement.

Perinetti et al.[22] (2002) investigated alkaline phosphatase (ALP) activity in GCF, to assess whether it can serve as a diagnostic aid in orthodontics. Sixteen patients

participated in the study. One first molar was the distalized molar (DM), whereas the contralateral molar (CM) was included in the fixed orthodontic appliance but was not subjected to the distal forces. The antagonist first molar (AM), free from any orthodontic appliance, was used as the baseline control. The GCF around the experimental teeth was collected from mesial and distal tooth sites immediately before appliance activation, 1 hour after, and weekly over the following 4 weeks. GCF ALP activity was significantly elevated in the DMs and the CMs as compared with the AMs at 1, 2, 3, and 4 weeks; conversely, in the AMs, GCF ALP activity remained at baseline levels throughout the experiment. Moreover, the enzyme activity in the DMs was significantly greater than in the CMs. In the DMs, a significantly greater ALP activity was observed in sites of tension compared with sites of compression.

Plagnat et al[58] (2002) studied ALP in GCF from implants with and without peri-implantitis and suggested that ALP could be a promising marker of bone loss around dental implants.

Smaro Kavadia[59] (2002) showed that during the course of orthodontic treatment, force produces distortion of the PDL extra cellular matrix, resulting in alteration in cellular shape and cytoskeletal configuration. These changes modify both GCF flow rate and its components. Therefore analysis of GCF sample provides a better understanding of biochemical processes associated with tooth movement.

Toms[60] (2002) stated that orthodontic forces by altering the blood flow and the localized electrochemical environment, upset the homeostatic environment of the

PDL space. This abrupt alteration initiates a biochemical and cellular cascade of events that reshapes the bony contour of the alveolus.

Andrew Delima et al[61] (2003) explained the origin and function of the cellular components in GCF. GCF was discovered in the late 1950s and 1960s by the experimental work done by Warehaug in dogs by introducing India Ink into gingival sulci. After 1 hour there was fluid transudation and emigration of leukocytes and within 48 hours, this transudate eliminated all the ink particles from sulci.

It was discovered that GCF can be isolated from both healthy sulci and diseased pockets. This fluid arises from the gingival plexus of blood vessels in the gingival corium, subjacent to the epithelium lining the dento-gingival space. It also contains desquamated epithelial cells, molecules originating from host tissues, sub-gingival plaque and oral bacteria.

Emanuela Serra et al[62] (2003) indicated possible role of GCF LDH during the early phases of orthodontic treatment in their study on 37 subjects while retracting a maxillary canine.

Griffiths[63] (2003) reviewed on the formation, collection and significance of Gingival Crevicular Fluid. He states that GCF is a transudate of interstitial fluid initially and on stimulation it is changed to an inflammatory exudate. He employed several techniques for collection of GCF: gingival washing methods, capillary tubing or micropipette methods and absorbent filter paper strips by either intra sulcular or extra sulcular methods.

GCF collection using micropipettes of known internal diameter can be used by placing it at the entrance of gingival crevice. A known volume of fluid can be accurately determined since the internal diameter is known. This method is an ideal method as it provides an undiluted sample of native GCF with known volume. However the disadvantage of this method is that it takes a very long duration to collect a reasonable volume. He also describes the problems encountered with GCF collection and data interpretation as variations in results with different sampling methods, chances of contamination, sampling time, volume determination and recovery from strips.

Marcin Balcerzak *et al*[64] (2003) explained the role of Annexins and Alkaline Phosphatase in mineralization process. Both these proteins are present inside the matrix vesicles of osteoblasts and helps in formation of hydroxyapatite crystals. Annexins are involved in calcium ion homeostasis and Alklaine Phosphatases are involved in phosphate homeostasis, inside the matrix vesicles which are necessary to initiate mineralization.

Max Goodson et al[65] (2003) says that an important characteristic of GCF is its flushing action. Substances put into the periodontal pocket are rapidly washed out. The GCF flow is about few microliters per hour.

Sugiyama et al[66] (2003) found that the accumulation of cathepsin B in GCF has been shown to increase with orthodontic tooth movement.

Takashi et al[67] (2003) found that MMP-1, 2, 3, 8, 9 and 13 were expressed in the PDL and alveolar bone during orthodontic tooth movement. He also found that the expression of MMP-8 and MMP-13 mRNA transiently increased in both tension and compression site during tooth movement.

Kee Joon Lee[68] (2004) evaluated the effects of light continuous force and interrupted force with weekly reactivation on interleukin-1 beta and prostaglandin E2 which are potent inflammatory mediators in tooth movement. They concluded that as for the duration of the orthodontic force, continuous force has better effects on tooth movement, whereas in terms of second messengers, intermittent forces were proven to have greater effects.

Harold Frost[69] (2004) stated that Osteoblasts and Osteoclasts are the key players in bone's physiology. Both these are controlled independently and increased osteoclastic activity cause bone loss and increased osteoblastic activity cause bone gains. Bio-chemical and genetic factors make these key players determine bone architecture, bone healing, size of bone bank and most bone disorders.

He put forth the famous Uttah Paradigm and Mechanostat theory to explain the bone's tissue level mechanism.

- The load bearing bones of human body such as maxilla, mandible, femur etc. are designed in such a way that the biologic machinery can adapt these bones to mechanical loads.
- This happens by two tissue level mechanisms: bone modeling and remodeling. Bone modeling causes formation and resorption drifts changing the shape and

size of bone and it strengthens the bone. Bone remodeling by the Basic Multi-cellular Units (BMUs) turns bone over in small packets.

- Loads on bone cause bone strains that generate signals that some cells can detect and to which they or other cells can respond.
- Genetically determine threshold ranges of these signals control bone modeling and remodeling.

Von Bohl[70] (2004) demonstrated the presence of Tartarate resistant Acid Phosphatase (TRAP) and alkaline phosphatase during the early stages of tooth movement in beagle dogs with the application of high and low forces.

Batra et al[21] (2005) investigated alkaline phosphatase activity in GCF during canine retraction using NiTi coil spring exerting 100g force in ten female patients requiring all first premolar extractions. Maxillary canine on one side acted as the test tooth and the other side canine was control tooth. GCF was collected before initiation of retraction, immediately after initiation of retraction, 1st, 7th, 14th, 21st day. They found that there was significant changes in ALP activity on 7th, 14th, 21st day between test and control tooth on both mesial and distal sites. The peak in enzyme activity occurred on the 14th day. They also found that enzyme activity varied according to the amount of tooth movement.

Laura R Iwasaki et al[71] (2005). Continuous maxillary canine retraction stresses of 13kPa and 4,26, or 52kPa were applied bilaterally in 6 growing and 4 adult subjects for 84 days. Dental models & GCF and stimulated whole blood samples were collected at 1- to 14- day intervals. They concluded that velocity of tooth translation

varied with growth status and stresses <52kPa which showed no lag phase. These correlated with cytokines in GCF and whole blood.

Guvenac Basaran et al[72] (2006) concluded that leveling and distalization of teeth evoke an increase in interleukin 2, 6 and 8 levels in the periodontal tissue.

Masaru Yamaguchi et al[73] (2006) showed that the amounts of SP and IL-1 beta in GCF increase with orthodontic tooth movement and indicate that such increase may be involved in the inflammatory response to mechanical stress.

Richard Masella[74] (2006) Adaptive biochemical response to applied orthodontic force is a highly sophisticated process. Many layers of networked reactions occur in and around periodontal ligament and alveolar bone cells that change mechanical force into molecular events (signal transduction) and orthodontic tooth movement (OTM). Osteoblasts and osteoclasts are sensitive environment-to-genome-to-environment communicators, capable of restoring system homeostasis disturbed by orthodontic mechanics. Five micro-environments are altered by orthodontic force: extracellular matrix, cell membrane, cytoskeleton, nuclear protein matrix, and genome. Hundreds of genes and thousands of proteins participate in OTM. Bone adaptation to orthodontic force depends on normal osteoblast and osteoclast genes that correctly express needed proteins at the right times and places. Cell membrane receptor-ligand docking is an important initiator of signal transduction and a discovery target for new bone-enhancing drugs. Inter-patient variation in mechanobiological response is most likely due to differences in periodontal ligament and bone cell populations, genomes, and protein expression patterns. Orthodontic treatment is likely to evolve into a

combination of mechanics and molecular-genetic-cellular interventions: a change from shotgun to tightly focused communication with OTM cells.

Sarandeep Huja *et al*[75] (2006) studied the remodeling dynamics of alveolar process in dogs. They showed that there are differences in remodeling rates between the maxillary and mandibular alveolar processes. Bone volume was 2.2 fold greater in mandible than maxilla. The bone formation rate was greater in mandible than maxilla. In the maxilla, anterior part had two fold greater remodeling rate than the posterior part but this was not seen in mandible.

Vinod Krishnan and Ze'ev Davidovitch[2] (2006) stated that remodeling changes in paradental tissues is an essential in effecting tooth movement. The force induced tissue strain produces local alterations in vascularity, as well as cellular and extra cellular matrix reorganization, leading to the synthesis and release of various neurotransmitters, cytokines, growth factors, colony stimulating factors and metabolites of arachidonic acid. This article reviews briefly the processes of bone, PDL and gingival remodeling in response to orthodontic force. It also provides insight into the biological background of various deleterious effects of orthodontic forces.

Ellis Golub[18] (2007) reviewed the role of alkaline phosphatase in hard tissue formation and mineralization. It catalyzes the hydrolysis of phosphomonoesters, R-O-PO3,with little regard to the identity of the 'R' group. The catalytic mechanism involves the formation of a serinephosphate at the active site which reacts with water at alkaline pH to release inorganic phosphate from the enzyme. This enzyme increases the local concentration of inorganic phosphate, which is a mineralization promoter

and decreases the concentration of extracellular pyrophosphate, which is a mineralization inhibitor. The enzyme is localized to the outside ofthe plasma membrane of cells, and of the membrane of matrix vesicles. It is attached to the membrane by a glycophosphatidylinositol anchor, and is found in membrane microdomains known as rafts. He states that measurement of increased ALP expression enzymatically, histochemically or at the mRNA level istaken as a reliable indication of the osteoblastic, chondrocyticor odontoblastic phenotype.

Henneman *et al*[77] (2008) explains on the mechanobiology of tooth movement. They explain the mechanical and biological signaling pathways during orthodontic tooth movement. The events taking place are divided into four stages: matrix strain and fluid flow, cell strain, cell activation and differentiation, and remodeling.

Dannan et al[78] (2009) studied the effect of orthodontic tooth movements, specifically canine retraction, on the volume of GCF exudates. They concluded that GCF volume at tension site was slightly greater between 21 & 28 days and at pressure site GCF volume was slightly greater after 28 days.

Lei Zhang *et* [a14l] (2009) explains the clinical utility of saliva and the salivary biomarkers in the diagnosis of periodontal disease and to assess the disease severity and the response to treatment.

Tomoko Kumasako[79] (2009) compared osteoclast recruitment and the extent of root resorption in response to an 8 hour intermittent force regimen with those from continuous force. The duration of force treatment is an important factor in optimizing

orthodontic tooth movement with less root resorption. Results show that an 8-hour intermittent force efficiently recruits osteoclasts while causing minimal root resorption.

Andrea Marcaccini et al[16] (2010) evaluated myeloperoxidase activity in GCF and saliva at different time intervals in humans with orthodontic fixed appliance activations. They found that MPO activity increased n both GCF and saliva at 2 hours after appliance activation. This may be because of the PMN infiltration into PDL that resulted in increased MPO activity at 2 hours. They suggested that MPO might be a good marker for inflammation in orthodontic tooth movement.

Hughes[80] (2010) stated that mechanical loading by orthodontic forces induce micro damage in the bone cells which acts as a stimulus for bone modeling and remodeling. Osteocytes exhibit mechanoreceptors that are sensitive to mechanical loading and micro damages. These micro damages in turn leads to apoptosis of osteocytes in the damaged regions.

Jonas Capelli et al[81] (2010) analyzed the gingival fluid volume during canine retraction using 150g continuous force at different time periods. He explains that the acute inflammatory process that characterizes initial stage of tooth movement is predominantly exudative, in which plasma and leukocytes migrate outside the capillaries in areas of paradental stress. After one or two days, the acute stage of inflammation is decreased and replaced by a chronic process involving fibroblasts, endothelial cells and osteoblasts. During this period, the leukocytes continue to migrate in the stressed paradental tissues and modulate a remodeling process.

Rahul Kathariya *et al*[13] (2010) reviews the literature on salivary proteomic constituents as potential biomarkers for oral diseases. He states that saliva offers a cost effective approach to assess oral diseases in large populations.

Teixeira et al[82] (2010) showed that inhibiting the expression of certain cytokines decreases tooth movement. This study hypothesized that stimulating the expression of inflammatory cytokines, through small perforations of cortical bone, increases the rate of bone remodeling and tooth movement.

Perinetti et al[83] (2010) found an increase in alkaline phosphatase levels during pubertal growth spurt and concluded that GCF ALP levels can be used as an adjunct in assessing the skeletal maturation & pubertal growth spurts in periodontally healthy subjects.

Antonio Hernandes Chaves Neto et al[84] (2011) investigated the serum and salivary levels of acid phosphatase, alkaline phosphatase and tyrosine phosphatase activity in 32 healthy children. He found that activities of all the enzymes were detectable in both serum and saliva and the activity was higher in serum than in saliva. Alkaline phosphatase activity in serum was 5.73 times higher than in saliva. He concluded that since there is correlation between the concentrations of saliva and serum and salivary enzymes can be used as biochemical markers of diseases in children.

Letitia *et al*[85] (2011) quantified the remodeling of bone surrounding primary teeth in skeletally immature dogs and compared it with existing studies on permanent teeth. He found that there was no difference in bone formation rates in primary and permanent dentitions and the bone formation rate was significantly higher in mandible when compared to maxilla. This is due to the fact that maxilla has smaller bone volume compared to mandible.

Nazeer Ahmed Meeran[8] (2011) reviewed the current knowledge on changes occurring in the GCF in response to orthodontic forces as well as the role of GCF in remodeling and adaptive changes in the paradental tissues during active tooth movement.

Randhir Kumar[15] (2011) quantitatively analyzed alkaline phosphatase activity in saliva in normal healthy gingiva, generalized gingivitis and generalized periodontitis patients. In healthy gingival the mean alkaline phosphatase levels were 18.5±5.07IU. The levels showed slight increase in gingivitis patients and a even higher increase in periodontitis patients indicating that alkaline phosphatase can be possible indicator of gingival inflammation and bone metabolism.

Sarah A et al[86] (2011) osteocalcin and N-telopeptides of type I collagen can be successfully estimated in the GCF and its increased levels might indicate the active tooth movement phase in orthodontic therapy.

Tina Pfaffe et al[87] (2011) describe the diagnostic potential of saliva and their uses. They explain the production of saliva, the biomolecules found in it, the various ways of transfer of biomolecules from blood to saliva and their collection methods.

Ildeu Andrade et al[88] (2012) explained the reaction of periodontal tissue to orthodontic force in both microscopic and macroscopic level leading to changes in 5 distinct environment: microenvironment, extracellular matrix, cytoskeleton, nuclear protein matrix and genome. They further explained that inflammatory process is a precondition for these modifications to occur and also changes in vascularity and blood flow in PDL causes the release of various key mediators such as chemokines, cytokines and growth factors. These molecules induce many cellular responses in the periodontium, providing a favourable environment for bone deposition or resorption leading to OTM.

Rodrigo Castellazzi *et al*[89] (2012) studied the changes in the periodontal ligament thickness on tension and compression sides during orthodontic tooth movement in rats. In his discussion he explains why rat models are used and how they are related to humans. Murine molars exhibit limited development so that the biological events that take place during orthodontic tooth movement are very similar to those of humans but occur in a shorter period of time since these animals have an accelerated metabolism.

Fabriziad'Apuzzo[10] (2013) lists the biomarkers of periodontal tissue remodeling during orthodontic tooth movement in mice and men. He classifies them as biomarkers of inflammation, biomarkers of cell death, biomarkers of bone resorption and biomarkers of bone deposition and mineralization.

Florez Moreno et al[17] (2013) evaluated the changes in soluble RANKL and OPG levels in saliva and their ratios during different phases of orthodontic tooth movement in 21 patients undergoing fixed appliance therapy. RANKL and OPG levels were determined using ELISA method and the results showed that RANKL levels increased and OPG levels decreased over time after the activation visit. Also their ratios tended to increase significantly with increase in time intervals. Hence these analytes might serve as a salivary biomarker in the screening of orthodontic treatment.

Jose Luis Millan[90] (2013) explains the role of Phosphatases in the initiation of mineralization. Mineralization begins inside the Matrix Vesicle (MV) of osteoblasts which serve as sites of calcium ion and inorganic phosphate accumulation to initiate the deposition of Hydroxyapatite crystals. These hydroxyapatite crystals are then released into the extra-cellular fluid and further deposition on to the collagenous extra-cellular matrix by rupture of MV. The control of these events are by the functional interplay of three phosphatases: Tissue Non-specific Alkaline Phosphatase, Phosphatase Orphan-1 (PHOSPHO1) and Nucleoside Pyrophosphohydolase-1 (NPP1). The mechanism of action of these three phosphatases and their interactions in maintaining the inorganic pyrophosphate to phosphate ratio (PPi/Pi) which is a key player in initiating skeletal tissue mineralization is explained in this article.

Alejandra Navarro *et al*[91] (2014) studied myeloperoxidase activity in GCF and whole saliva in patients with different levels of dental crowding during aligning period. The enzyme values increased after 2 hours and remained elevated till 7 days after which there was fall to baseline values in both GCF and saliva. Though the

pattern of enzyme was same in GCF and saliva, GCF showed more accurate enzyme activity than saliva. The values of myeloperoxidase in saliva was 10 fold higher than GCF due to the dilution factor in GCF.

Mikulewicz *et al*[92] (2014) in an in-vitro study done for 28 days found that there is release of metal ions from orthodontic appliances into saliva. The release of ions is in the order of Si>Cu>Ni>Cr>Mo>Mn>Cd. The total mass of released metal ions from the appliance during 4 weeks of the experiment was as follows: nickel 18.7 mg, chromium 5.47 mg, copper 31.3 mg. The ions were released in doses not toxic to humans.

MATERIALS&METHODS

MATERIALS USED IN THE STUDY:

1. Mouth mirror, Probe, Tweezer
2. Cheek retractor, Cotton rolls
3. William's periodontal probe
4. Universal Gracey curette
5. NiTi coil springs
6. Dontrix gauge
7. Drummond PCR micropipettes (1-10 microlitre) & plungers
8. 50ml Sterile Falcon tube labeled
9. Sterile Plastic vials labeled
10. Transporting thermosealed ice box (Cello)
11. Coolant gel packs (Biosystems) and dry ice
12. Glacial Acetic acid (Merck Specialities)
13. -40 degree Celsius storage freezer (Thermo Forma -87C ULT freezer)
14. Micro-centrifuge (REMI cooling centrifuge)
15. Auto Analyser (Merck Micro Lab 300)
16. Phosphate Buffered Saline (pH 7.4)
17. Innoline Alkaline phosphatase assay reagents (Merck Micro Lab)
18. Cyclomixer
19. Nikon D 300S camera

METHODOLOGY:

The present study was undertaken in the Department of Orthodontics and Dento-facial Orthopedics, Sri Ramakrishna Dental College and Hospital, Coimbatore. The patients who participated were explained about the study and informed consent forms were obtained from them. This study was reviewed and approved by the Ethical Committee of this college.

STUDY SAMPLE:

A total of ten patients, in the age group between 18 to 21 years, requiring fixed appliance therapy with extraction of first premolars as a part of their treatment plan were selected to participate in this study. All the patients had Angle's class I malocclusion with minimum or no crowding as assessed by PAR Index displacement scores of less than or equal to 1[92].

SELECTION CRITERIA:

1. Good general health with no systemic diseases, assessed after careful history taking.
2. Good oral health and hygiene with an Oral Hygiene Index score good and gingiva showing no signs of inflammation as assessed by gingival index score less than 1.
3. Good periodontal health with periodontal probing depth not more than 3mm and showing no radiographic evidence of alveolar crestal bone loss.
4. Patient not under any antibiotics or anti-inflammatory drugs for the last three months.

5. Female patients were ruled out for pregnancy or lactation.
6. Smokers were excluded from the study.

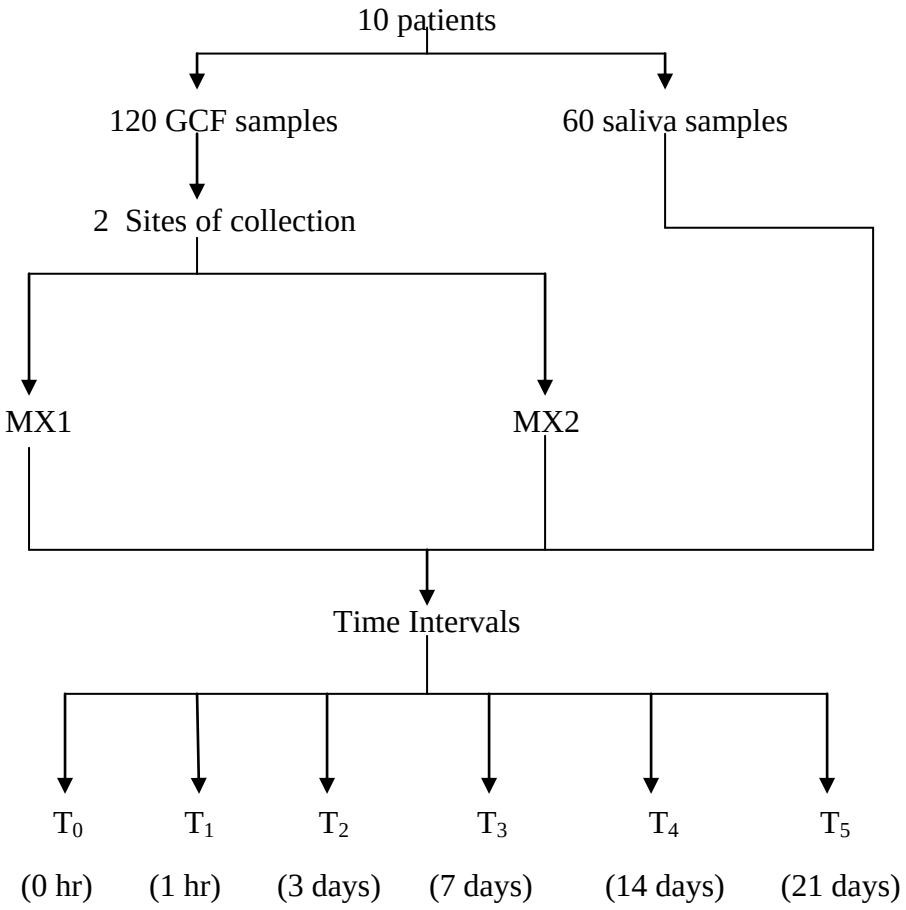
PATIENT PREPARATION:

All the ten patients were given repeated oral hygiene instructions on the use of tooth brush and 5ml of 0.2% Chlorhexidine mouth rinse was used twice daily. The patients underwent thorough oral prophylaxis three weeks prior to the study. Oral Hygiene Index- Simplified (OHI-S) and Gingival Index were taken regularly throughout the study period to assess the gingival condition of patients and to rule out the possibility of inflammation biasing the results of our study. Prior to sample collection, probing depth was measured using the William's periodontal probe to rule out periodontitis, which may also lead to an increase in the alkaline phosphatase assay values creating a bias in our study (Figure:7). The patients were not allowed to take any antibiotics or anti-inflammatory drugs during the period of study to avoid interference in the sterile inflammatory process associated with orthodontic tooth movement.

STUDY DESIGN:

For all the ten patients, Saliva samples and Gingival Crevicular Fluid (GCF) samples were collected at baseline (T_0), that is, before force application for en masse retraction and with force application at 1 hour (T_1), 3 days (T_2), 7 days (T_3), 14 days (T_4) and 21 days (T_5). Two sites were chosen for GCF collection, they are the gingival sulcus in relation to the mesio-labial line angle of right maxillary canine which is designated as MX1 and the gingival sulcus in relation to the mesio-labial line angle of left maxillary canine which is designated as MX2. Thus, 12 GCF samples and 6 saliva

samples were collected for each patient. Hence, a total of 120 GCF samples and 60 saliva samples were collected for all the ten patients.



ORTHODONTIC MECHANOTHERAPY:

All the ten patients underwent treatment with MBT straight wire appliance with 0.022”x0.028” slot brackets. First premolar extractions were carried out at the beginning of treatment and the duration for leveling and aligning to be completed for all the samples were between 6 months to 8 months. This study has been designed to assay the Alkaline Phosphatase enzyme activity during en masse retraction in the maxillary arch. Hence after leveling and aligning, 19” x 25” stainless steel wire was placed in the maxillary arch for a period of four weeks, during which time the patients

were under strict oral hygiene maintenance regime as described above. After four weeks, J hooks were soldered on to the 19" x 25" stainless steel wire and en masse retraction was carried out using NiTinol closed coil springs delivering about 150 grams of continuous force as measured with a Dontrix gauge[47,93].

SAMPLE COLLECTION:

The GCF and saliva samples for all the ten patients were collected before the mid-day meal and either before breakfast or two hours after breakfast[94]. Saliva should be collected before performing any procedure in the patient's oral cavity. Hence, saliva samples were collected always before GCF sample collection. The patients were asked to rinse the mouth with water prior to sample collection. Patients were seated comfortably in an upright position and were asked not to swallow the saliva that was passively getting collected in their mouth. This unstimulated whole saliva, when sufficient quantity got pooled in their mouth, was spit into a sterile falcon tube[94] which was labeled with the patient's name and the time interval of collection (T0, T1, T2, T3, T4, T5) (Figure:5). Approximately 2ml of saliva was collected, the tube was sealed and transferred to the thermosealed cooler box containing coolant gel packs which were frozen to -20° C.(Figure:3).

Before collecting GCF samples, patient's oral cavity was isolated properly with cheek retractor and cotton rolls. Any debris present at the site of collection of GCF was removed with a curette (Figure:6), being careful to avoid bleeding and the area was dried with a gentle stream of air for 5 seconds to prevent saliva contaminating the GCF[20]. An extra-sulcular method of collection of GCF using volumetric micropipettes of 1 microlitre capacity was used in our study (Figure:2). An advantage of using micropipettes over Periopapers or threads is that, a Periotron is not

required to measure the quantity of GCF collected, since the micropipettes are pre-calibrated on a 1 microlitre scale. This method is less technique sensitive, easier to collect, predetermined volumes are collected and no special buffers are needed to extract the fluid from the absorbent papers or threads during analysis[63].

The micropipette was placed extra-sulcularly in the mesio-labial line angle of maxillary canine tooth for about 15 minutes or until 1 microlitre GCF volume was collected[21] (Figure:8). Samples contaminated with blood or debris were discarded and collected again. The micropipette was then placed in labeled sterile plastic vials and transferred to the thermosealed cooler box with coolant gel packs frozen to -20°C (Figure: 3). The collected samples were taken to the laboratory where the GCF samples were diluted with 100 microlitres of phosphate buffered saline (pH 7.4) in a sterile plastic vial (Figure: 9, 10) and stored at - 40°C after adding a drop of glacial acetic acid stabilizer into it[21] (Figure: 11, 12). The saliva samples were also stored at -40°C after adding a drop of glacial acetic acid stabilizer. By the above mentioned methods, saliva and GCF samples were collected before force application, 1 hour, 3 days, 7 days, 14 days and 21 days after force application in all the ten patients. The samples were stored for a period of 4 weeks after which it was assayed for alkaline phosphatase in the laboratory[27].

ALKALINE PHOSPHATASE ASSAY:

The stored samples are transported to the main laboratory kept inside an ice box with dry ice fully packed and coolant gel packs inside to maintain the frozen temperature (Figure: 5). The frozen samples were thawed for about 10 hours to bring it to room temperature[27]. Then the salivary samples were transferred to small sterile plastic vials using a pipette (Figure: 13) and they were centrifuged for 10 minutes at

10000 rpm to get a supernatant solution free of any debris (Figure: 14, 15). 800 microlitres of reagent R1 and 200 microlitres of reagent R2 of the Innoline alkaline phosphatase assay kits (based on DGKC and SCE kinetic method) were taken in a vial and after 25 seconds (Figure: 16, 17), 20 microlitres of supernatant solution of saliva was added and mixed in a Cyclomixer for 1 minute (Figure: 18, 19). This solution was then assayed for alkaline phosphatase levels Spectrophotometrically at 405nm wavelength and incubated at 37° C using a fully automated Auto Analyser (Merck Micro Lab 300). The Auto Analyser is calibrated to give the readings as IU/L (International Units per Litre) (Figure: 20).

Similarly, 20 microlitres of the diluted GCF solution was also mixed with alkaline phosphatase assay reagents, R1 and R2, mixed in Cyclomixer for 1 minute and assayed Spectrophotometrically using Auto Analyser to give the readings as IU/L.

PRINCIPLE OF ALKALINE PHOSPHATASE ASSAY:

The method of evaluation of the enzyme Alkaline Phosphatase using the Innoline ALP assay kit (Merck Laboratories) (Figure: 4) is a kinetic method based on DGKC (German Society of Clinical Chemistry) and SCE (Scandinavian Society of Clinical Chemistry) recommendations.

The composition of the Alkaline Phosphatase assay kit:

Reagent 1: R1 (Buffer)

Diethanolamine, pH 10.2	1.4 mol/L
Magnesium Chloride	0.625 mmol/L

Reagent 2: R2 (Substrate)

p-Nitrophenylphosphate	50 mmol/L

In the presence of Magnesium Chloride ($MgCl_2$) and Diethanolamine (DEA) buffers as phosphate acceptors, p-Nitrophenylphosphate (substrate) is hydrolysed by the enzyme, Alkaline Phosphatase into inorganic Phosphate and p-Nitrophenol (yellow compound) at pH 10.2.

$$\text{p-Nitrophenyl phosphate} + H_2O \xrightarrow{\text{ALP}} \text{p-Nitrophenol} + \text{Phosphate}$$

The rate of p-Nitrophenol formation, measured Spectrophotometrically at 405nm and 37°C, is proportional to the catalytic concentration of alkaline phosphatase present in the sample. 1 unit of Alkaline Phosphatase activity is 1µmol of p-nitrophenyl phosphate converted to p-nitrophenol and inorganic phosphate per minute at 37°C and at pH 10.2.

The Auto Analyser measures the changes in absorbance of yellow colour (p-Nitrophenol) per minute (ΔA/min) for 3 minutes.

Activity (IU/L) = Δ A/min. X 2750

AUTO ANALYSER PARAMETERS :

Mode	Kinetic
Wavelength	405nm
Sample vol. µl	20
Reagent vol. µl	1000
Delay time sec.	60
Read time sec.	180
Factor/Stnd.	2750
Linearity	700 IU/L
Temp.	37°C
Unit	IU/L
Ref. Low	0
Ref. High	270

CONSENT FORM

I, Mr/Mrs/Ms..... Aged ..... was made aware by the doctor about the study that involves the collection of my Saliva and Gingival Crevicular Fluid to find the biomarker, Alkaline Phosphatase during orthodontic tooth movement. I was also explained about the duration of the study and my responsibility in keeping up my appointments and maintaining my oral hygiene as instructed by the attending orthodontist. I give my consent to use my records for educational purposes in articles or books. I agree to participate in this study and give my full consent for the treatment procedures.

Date:

Place: Patient signature

PATIENT'S PROFORMA

PATIENT'S NAME :

GENDER / AGE :

OP NO :

STARTED ON :

PG STUDENT :

STAFF IN-CHARGE :

CHEIF COMPLAINTS:

MEDICAL HISTORY:

DENTAL HISTORY

INTRA-ORAL EXAMINATIONS:

1. ORAL HYGIENE STATUS ASSESMENT

ORAL HYGIENE INDEX- SIMPLIFIED

DEBRIS: 16 11 26 36 31 46

SCORE:

GOOD/ FAIR/ POOR

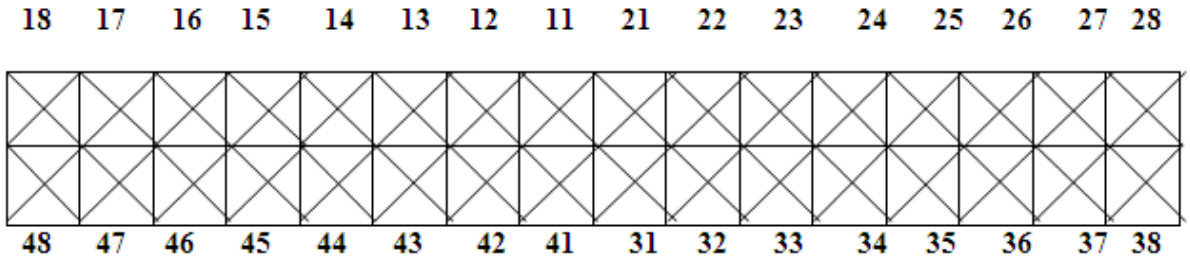
CALCULUS:

16 11 26 36 31 46

SCORE:

GOOD/ FAIR/ POOR

GINGIVAL INDEX:



SCORE:

PAR Index Displacement scores:

DATE & TIME OF SAMPLE COLLECTED:

STAFF SIGNATURE:

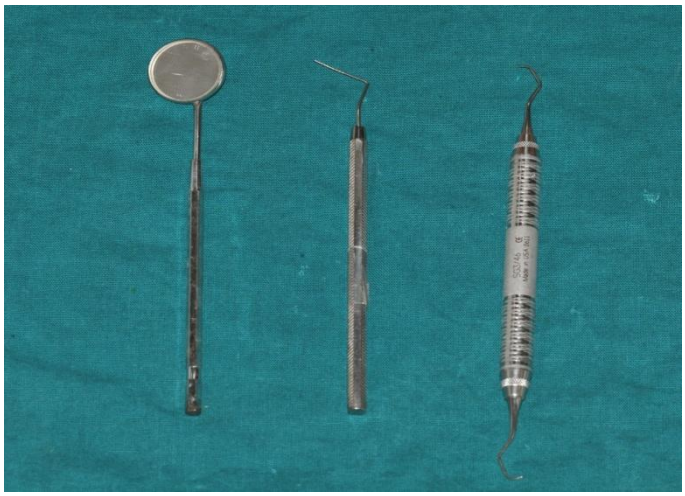


Fig 1: Mouth Mirror, William's periodontal probe, Universal Gracey Currette

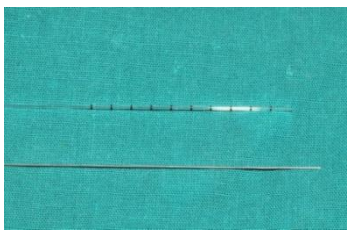


Fig 2: Collection medium: 50ml sterile Falcon tube, Drummond PCR Micropipets(1-10µl) with plungers, sterile plastic vials labeled.

Fig 3: Transporting medium: Thermosealed ice box, dry ice, coolant gel packs

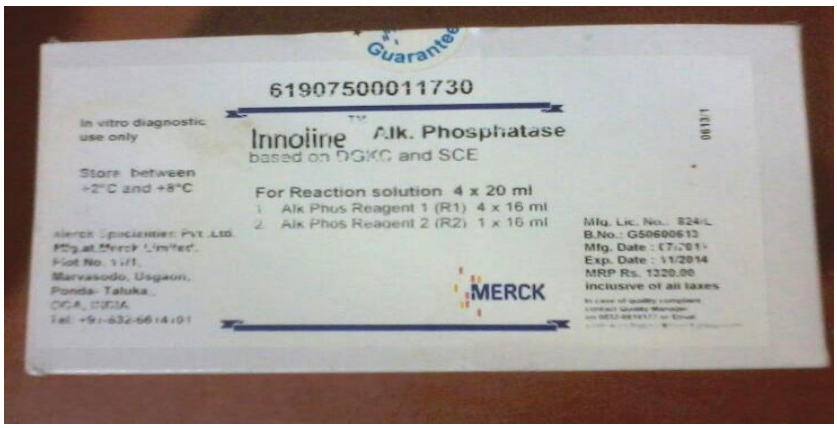


Fig 4: Alkaline Phosphatase Assay enzyme kit

Fig 5: Collection of unstimulated whole saliva

Fig 6: Removal of soft Debris and plaque with curette

Fig 7: Checking for Probing depth with William's Periodontal probe

Fig 8: Collection of GCF: Micropipettes placed extra-crevicularly at the mesio-labial line angles of canine till 1µl GCF got collected

Fig 9: 100µl buffered saline taken in a sterile plastic vial using pipette

Fig 10: GCF expelled into saline using the plunger

Fig 11: A drop of glacial acetic acid added for stabilization

Fig 12: Diluted GCF and collected saliva stored at -40°C

Fig 13: Saliva transferred to sterile plastic vials using pipette

Fig 14: Saliva centrifuged at 10000 rpm for 10 minutes in REMI microcentrifuge

Fig 15: After centrifugation, Supernatant solution, free of debris collected at top

Fig 16: 800µl of reagent R1 taken in a sterile plastic vial

Fig 17: 200µl of reagent R2 added

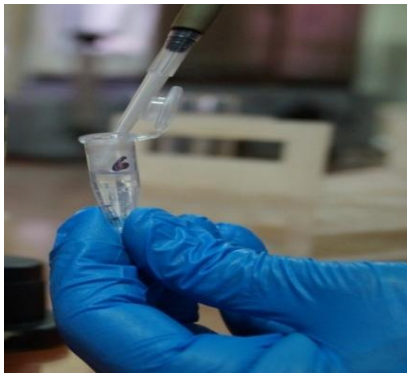


Fig 18: 20µl of diluted GCF/ Saliva added to the reagents

Fig 19: Contents mixed in a Cyclomixer for 1 minute

Fig 20: The solution placed for Alkaline Phosphatase assay in an Auto Analyser(Merck Micro Lab 300). The values are displayed at the LCD screen.

RESULTS

A total of ten patients in age group of 18 to 21 years with Angle's class I malocclusion requiring first premolars extraction were selected. For each patient GCF samples were collected from two sites: the gingival sulcus in relation to the mesio-labial line angle of right maxillary canine and the gingival sulcus in relation to the mesio-labial line angle of left maxillary canine, and saliva samples were also collected at base line, 1 hour, 3 days, 7 days, 14 days and 21 days after force application. Thus, 12 GCF samples and 6 saliva samples were collected for each patient. Hence, a total of 120 GCF samples and 60 saliva samples were collected for the study.

STATISTICAL ANALYSIS:

One-Sample Kolmogorov-Smirnov Test was done to show that the test samples followed a Normal or Gaussian distribution. Since the test distribution was Normal, Paired Samples't' Test was done to compare the Alkaline Phosphatase activity in GCF (MX1 and MX2) and saliva separately, within each time interval. Growth Rates of Alkaline Phosphatase activity at each time intervals in GCF and saliva were calculated and Paired Samples't' Test was done to compare the compounded growth rate in GCF and saliva.

PARAMETERS ASSESSED:

1. Changes in Alkaline Phosphatase level in GCF and saliva at different time intervals.
2. Pattern of rise of Alkaline Phosphatase activity in GCF and saliva at different time intervals
3. Comparison of Alkaline Phosphatse activity in GCF within different time intervals in both the maxillary sites, MX1 and MX2.

4. Comparison of Alkaline Phosphatse activity in saliva within different time intervals.
5. Comparison of Growth Rates of Alkaline Phosphatase in GCF and saliva.

1. Changes in Alkaline Phosphatase level in GCF and saliva at different time intervals:

Mean values of each time interval Alkaline Phosphatase level in each site of GCF and saliva was calculated with their respective standard deviations. There is an increase in Alkaline Phosphatase levels from baseline to 21st day in both the maxillary sites in GCF as shown by the mean values in Table:1. An increase in Alkaline Phosphatase levels from baseline through the 21st day is also seen in saliva. There is only a very mild increase from baseline (T_0) to one hour after force application (T_1). A marginal increase in enzyme levels is seen on the 3rd day (T_2). A steep increase in Alkaline Phosphatase level is noted on the 7th day (T_3) followed by increase in levels on the 14th day (T_4) in both GCF and saliva. There is a mild increase in enzyme levels on the 21st day (T_5).These are shown by the mean values of Alkaline Phosphatase levels at different time intervals in both GCF and saliva in Table:1 and Graph:1.

2. Pattern of rise of Alkaline Phosphatase activity in GCF and saliva at different time intervals:

The incremental changes in Alkaline Phosphatase levels at different time intervals, as shown in Table: 2, is calculated by finding the mean difference in enzyme levels from one time interval to the previous time interval in both the sites of GCF and saliva. This shows that only a very minimal activity of Alkaline Phosphatase is seen from T_0 to T_1 in both GCF and saliva. A significant amount of increase in

enzyme activity is seen from T_1 to T_2 in both GCF and saliva. A Peak in rise of Alkaline Phosphatase activity is seen during the time interval from T_2 to T_3 in both GCF and saliva. There is a slight decline in the amount of increase of enzyme activity from T_3 to T_4. After T_4 there is a marked decline in rise of Alkaline Phosphatase activity in both GCF and saliva till T_5. The pattern of enzyme activity in GCF and saliva based on these incremental changes is shown in Graph: 2.

3. **Comparison of Alkaline Phosphatase activity in GCF within different time intervals in both the maxillary sites, MX1 and MX2:**

Paired Samples 't' Test shows that in both MX1 and MX2, the increase in Alkaline Phosphatase activity between T_0-T_1 and T_4-$T_5$was less significant (p values >0.01 but <0.05) but the increase in alkaline Phosphatase activity between T_0-T_2, T_0-T_3, T_0-T_4, T_0-T_5, T_1-T_2, T_1-T_3, T_1-T_4, T_1-T_5, T_2-T_3, T_2-T_4, T_2-T_5, T_3-T_4, T_3-T_5 were highly significant (p values <0.01). This is shown in Table: 3.

4. **Comparison of Alkaline Phosphatse activity in saliva within different time intervals:**

Paired Samples 't' Test for comparing the increase in Alkaline Phosphatase activity within different time intervals in saliva shows that the increase in activity between T_0-T_1 is not significant (p value > 0.05). The increase in activity between all other time intervals, T_0-T_2, T_0-T_3, T_0-T_4, T_0-T_5, T_1-T_2, T_1-T_3, T_1-T_4, T_1-T_5, T_2-T_3, T_2-T_4, T_2-T_5, T_3-T_4, T_3-T_5, T_4-T_5 were highly significant (p values <0.01) as shown in Table: 4.

5. Comparison of Growth Rates of Alkaline Phosphatase in GCF and saliva:

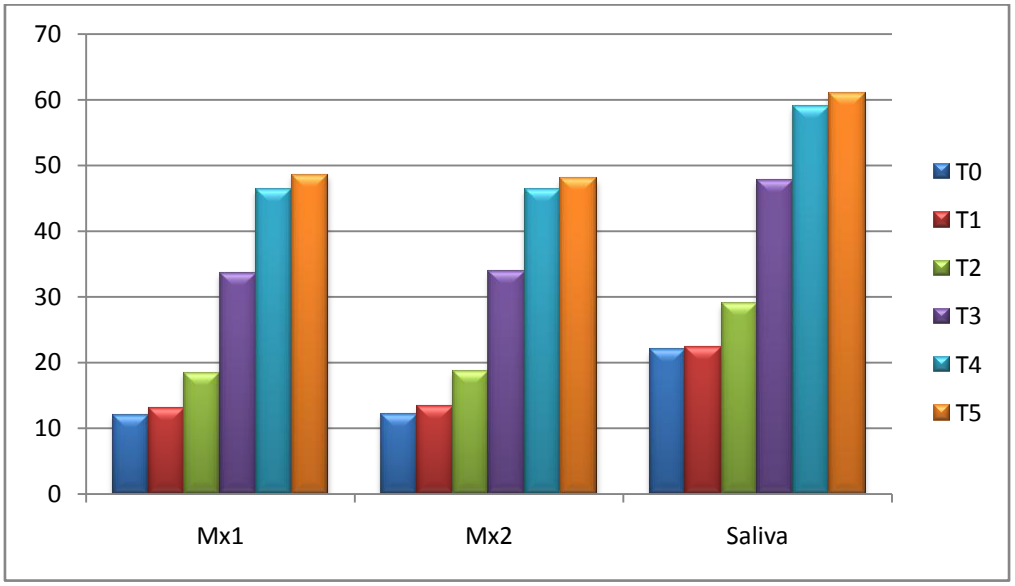
Growth Rates for Alkaline Phosphatase activity at each time interval ($T_0 \rightarrow T_1$, $T_1 \rightarrow T_2$, $T_2 \rightarrow T_3$, $T_3 \rightarrow T_4$, $T_4 \rightarrow T_5$) was calculated for both GCF and saliva (Table: 5). The growth rate of the enzyme was maximum at $T_2 \rightarrow T_3$ in both GCF and saliva. The growth rates were similar in the time intervals $T_0 \rightarrow T_1$ and $T_4 \rightarrow T_5$. The growth rates for all the time interval was less in saliva compared to GCF. The results are shown in Table: 5 and Graph: 3. To check the statistical significance of this difference in growth rates between GCF and saliva, a compounded growth rate of all the time interval was calculated and Paired Samples 't' Test was done (Table: 6). The results show that there is no significant difference in compounded growth rates between MX1 and MX2 (p value>0.05) but the difference in compounded growth rates between MX1- saliva and MX2- saliva is highly significant (p value< 0.01). The compounded growth rate is less in saliva compared to GCF as shown in Graph: 4.

Table: 1 Total Alkaline Phosphatase levels

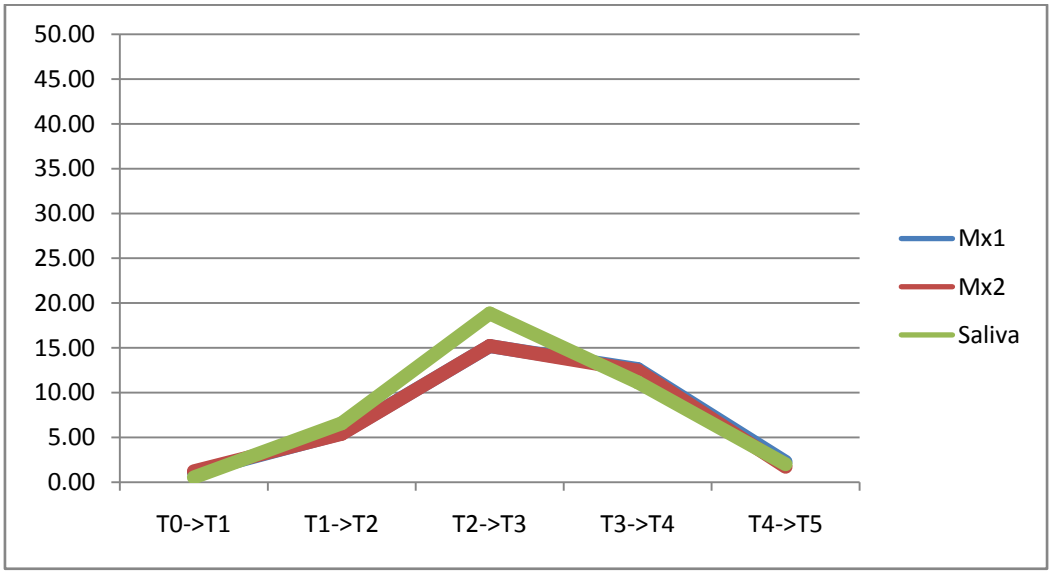
Time	N	Minimum	Maximum	Mean	Std. Deviation	Mean±SD
T0-Mx1	10	6.8	21.4	12	4.46	12±4.46
T1-Mx1	10	6.9	24.2	13.05	4.94	13.05±4.94
T2-Mx1	10	10.4	27.1	18.49	5.71	18.49±5.71
T3-Mx1	10	21.76	55.31	33.68	10.11	33.68±10.11
T4-Mx1	10	33.3	69.8	46.32	11.57	46.32±11.57
T5-Mx1	10	37.2	70.7	48.60	11.30	48.60±11.30
T0-Mx2	10	6.3	20.2	12.10	4.13	12.10±4.13
T1-Mx2	10	6.5	21.6	13.31	4.42	13.31±4.42
T2-Mx2	10	10.16	27.8	18.72	6.23	18.72±6.23
T3-Mx2	10	23.82	54.2	33.93	10.11	33.93±10.11
T4-Mx2	10	32.06	71.24	46.38	12.17	46.38±12.17
T5-Mx2	10	37.06	70.36	48.09	10.95	48.09±10.95
T0-Saliva	10	13.1	31	21.89	4.88	21.89±4.88
T1-Saliva	10	12.45	29.8	22.43	4.78	22.43±4.78
T2-Saliva	10	18.5	34.9	29.02	5.09	29.02±5.09
T3-Saliva	10	32.6	63.3	47.85	9.94	47.85±9.94
T4-Saliva	10	41	79.01	59.00	11.72	59±11.72
T5-Saliva	10	44.9	79.49	61.00	10.73	61±10.73

Table: 2 Incremental Changes in Alkaline Phosphatase activity at different time intervals

	N	Minimum	Maximum	Mean	Std. Deviation	Mean±SD
MX1 T0->T1	10	0.1	2.8	1.05	1.04	1.05±1.04
MX1 T1->T2	10	1.7	9.9	5.44	2.80	5.44±2.8
MX1 T2->T3	10	6.36	28.21	15.19	6.10	15.19±6.1
MX1 T3->T4	10	5.7	31.64	12.64	7.22	12.64±7.22
MX1 T4->T5	10	-0.5	6.47	2.28	2.29	2.28±2.29
MX2 T0->T1	10	0.12	3.85	1.21	1.24	1.21±1.24
MX2 T1->T2	10	1.62	9.8	5.40	2.82	5.4±2.82
MX2 T2->T3	10	8.31	26.4	15.21	5.46	15.21±5.46
MX2 T3->T4	10	7.13	27.86	12.45	6.33	12.45±6.33
MX2 T4->T5	10	-0.88	6.04	1.71	2.01	1.71±2.01
Saliva T0->T1	10	-1.2	2.25	0.55	0.97	0.55±0.97
Saliva T1->T2	10	3.93	11.42	6.58	2.27	6.58±2.27
Saliva T2->T3	10	7.07	32.17	18.84	8.08	18.84±8.08
Saliva T3->T4	10	6.92	20.72	11.15	4.41	11.15±4.41
Saliva T4->T5	10	0.48	4.47	2.00	1.39	2±1.39



Graph: 1 Increase in ALP levels at different time intervals



Graph: 2 Pattern of rise of ALP activity at different time intervals

Table: 3 Comparison of p values for ALP activity in both the sites of GCF within different time intervals

MX1	p value				
	T0	T1	T2	T3	T4
T1	0.012				
T2	0.000	0.000			
T3	0.000	0.000	0.000		
T4	0.000	0.000	0.000	0.000	
T5	0.000	0.000	0.000	0.000	0.045

MX2					
	T0	T1	T2	T3	T4
T1	0.010				
T2	0.000	0.000			
T3	0.000	0.000	0.000		
T4	0.000	0.000	0.000	0.000	
T5	0.000	0.000	0.000	0.000	0.025

Table: 4 Comparison of p values for ALP activity in saliva within different time intervals

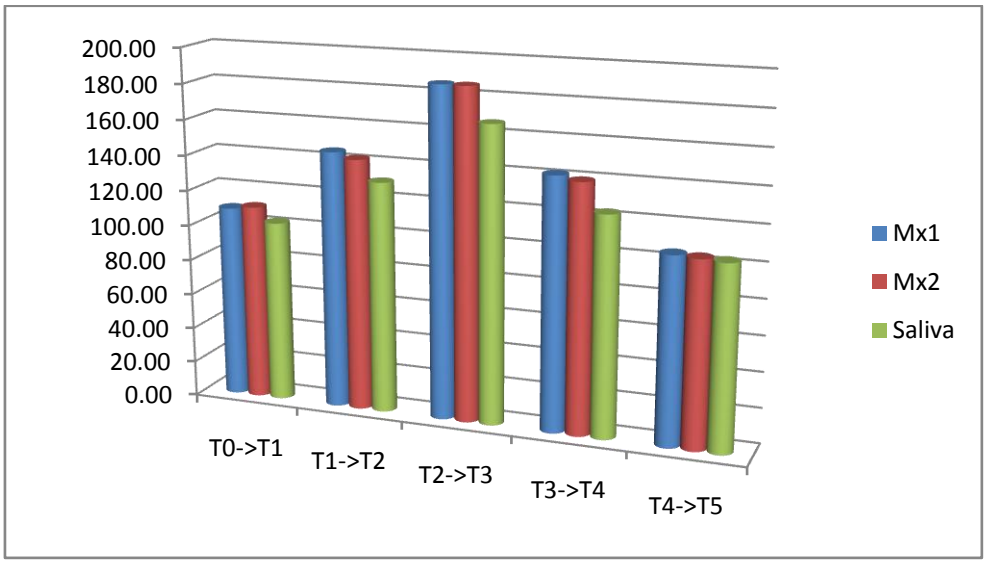
Saliva	p value				
	T0	T1	T2	T3	T4
T1	0.103				
T2	0.000	0.000			
T3	0.000	0.000	0.000		
T4	0.000	0.000	0.000	0.000	
T5	0.000	0.000	0.000	0.000	0.007

Table: 5 Growth Rates of Alkaline Phosphatase at each time interval

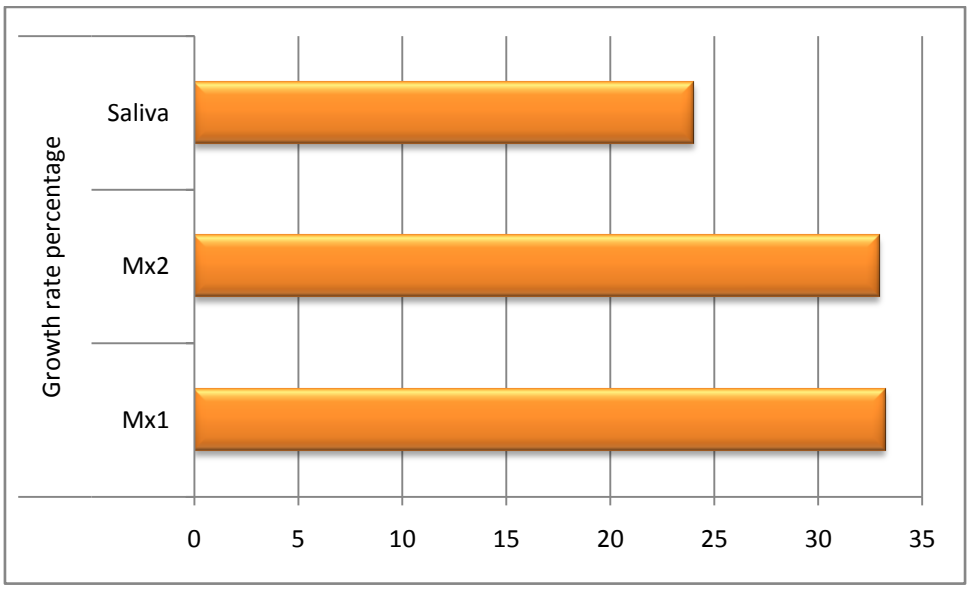
	N	Minimum	Maximum	Mean	Std. Deviation	Mean±SD
MX1 T0->T1	10	100.63	125.79	108.92	8.42	108.92±8.42
MX1 T1->T2	10	111.98	171.70	145.53	22.16	145.53±22.16
MX1 T2->T3	10	135.69	237.50	186.16	32.17	186.16±32.17
MX1 T3->T4	10	116.47	245.40	142.21	36.88	142.21±36.88
MX1 T4->T5	10	99.16	117.72	105.39	5.62	105.39±5.62
MX2 T0->T1	10	101.00	133.05	110.63	11.04	110.63±11.04
MX2 T1->T2	10	112.82	161.57	142.28	19.75	142.28±19.75
MX2 T2->T3	10	135.34	235.24	186.09	29.03	186.09±29.03
MX2 T3->T4	10	119.66	214.65	139.81	27.47	139.81±27.47
MX2 T4->T5	10	98.76	118.84	104.58	5.76	104.58±5.76
Saliva T0->T1	10	96.13	109.00	102.66	4.02	102.66±4.02
Saliva T1->T2	10	115.10	152.43	130.74	12.04	130.74±12.04
Saliva T2->T3	10	121.30	220.54	166.95	32.11	166.95±32.11
Saliva T3->T4	10	111.63	149.23	123.80	9.94	123.8±9.94
Saliva T4->T5	10	100.61	109.51	103.85	3.10	103.85±3.1

Table: 6 Paired Samples‘t’ Test for compounded growth rate in GCF and saliva

		95% Confidence Interval of the Difference				
		Lower	Upper	t	df	p values
Pair 1	Compounded growth rate Mx1 - Saliva	4.1600	14.2689	4.062	9	.002
Pair 2	Compounded growth rate Mx2 - Saliva	4.6509	13.2025	4.652	9	.001
Pair 3	Compounded growth rate Mx1 - Mx2	-1.8596	2.4351	.299	9	.771



Graph: 3 Growth Rates of GCF and saliva at each time interval



Graph: 4 Compounded Growth Rate in GCF and saliva

DISCUSSION

The biomechanical response of alveolar bone to orthodontic tooth movement involves an integrated process of bone modeling and bone remodeling. According to **Harold Frost**, bone modeling is an uncoupled sequence of bone resorption and formation occurring at two different sites and controlled independently, resulting in change in shape or size of bone and drifting of bone. The bone resorption on the pressure side and bone formation on the tension side seen during orthodontic tooth movement are commonly referred as bone modeling events. Bone remodeling, on the other hand, is a specific coupled turnover sequence (A→R→F) of cell activation, resorption of a cavity and replacement by formation of new bone. Here bone resorption and formation occur on the same sites without any change in the overall form of bone and helps to repair an area of defect and maintain the calcium homeostasis. When teeth are moved greater distances, such as those during en-masse retraction, the alveolar bone modeling is supported by bone remodeling events of both bone and PDL to maintain the physiologic internal turnover of alveolar bone and replace the woven bone formed by modeling process into mature lamellar bone[69,95,96]. **King and Keeling** stated that bone remodeling occur at both pressure and tension sites depending on the amount of bone formation and resorption occurring at each site as a result of bone modeling[43].

Orthodontic tooth movement is a sterile inflammatory type of reaction. A sustained force of upto 4 hours is necessary for the inflammatory mediators to initiate the differentiation and proliferation of the cells necessary for bone resorption, Osteoclasts and the cells for bone formation, Osteoblasts[97]. On the tension side, osteogenic response is noted in about 16 hours during which there is intense proliferative activity and new bone formation is initiated in about 40 to 48 hours[98].

The process of bone remodeling occurs in small packets of cells called Basic Multicellular Units (BMUs) consisting of a team of osteoclasts in front forming the cutting cone, a team of osteoblasts behind forming the filling cone, some form of blood supply and connective tissue[98]. **P.A Hill** explains that the remodeling process has four phases: Activation phase (osteoclastic precursors get activated and differentiates into mature osteoclasts in the presence of numerous inflammatory mediators and osteoblasts), Resorption phase (3-5 days), Reversal phase (5-7 days) and Formation phase (7-14 days). Mineralization of newly formed bone continues after 14 days[46]. Considering these phases, the time interval for this study (baseline, 1 hour, 3 days, 7 days, 14 days and 21 days) was chosen. **Carlalberta Verna *et al*** states the reason for choosing a study period of 21 days for most bone turnover studies as a complete remodeling cycle in a mature rat is 21 days[52]. **Rodrigo Castellazzi *et al*** states that the biological reactions that take place during orthodontic tooth movement in rats are very similar to those of humans but occur in a shorter period of time since these animals have an accelerated metabolism[88]. In a human remodeling cycle, the formation phase is shown to extend for a period of 2 to 3 months[51].

The cascade of events taking place in the alveolar bone in response to orthodontic force can be understood by studying the various enzymes and molecules participating in these modeling and remodeling processes which are called as Biomarkers of tooth movement. Of the various Biomarkers available, Alkaline Phosphatase is an enzyme that is very closely associated with bone formation and mineralization[10]. **Jonathan Sandy *et al*** states that osteoblasts are the cells that control both the resorptive and formative phase, in both bone modeling and remodeling

processes[42]. Osteoblasts are rich in Alkaline Phosphatase and it is associated with the matrix vesicles of the plasma membrane. It helps in initiating the mineralization process by maintaining the proper inorganic pyrophosphate to phosphate ratio (PPi/Pi). Inorganic phosphates are essential for the deposition of hydroxyapatite crystals whereas inorganic pyrophosphate is an inhibitor of mineralization. Alkaline Phosphatase catalyzes the hydrolysis of inorganic pyrophosphates to phosphates making them readily available for mineralization[58,90].

GCF has proved to be an effective medium to analyze the Biomarkers as they directly reflect the biochemical changes taking place in the PDL and alveolar bone[9]. Saliva is another oral fluid which can be collected in an easy and inexpensive way. According to **Frodge *et al***, the different mediators involved in alveolar bone remodeling are washed into the saliva by the Gingival Crevicular Fluid and hence whole saliva can be used to assess phase specific changes taking place during alveolar bone turnover and orthodontic tooth movement[99]. Hence these two potential oral fluids were chosen as the non-invasive medium to analyze Alkaline Phosphatase activity during en-masse retraction in this study.

Gao et al found that alkaline phosphatase activity was highest in osteoblasts, moderate in periodontal ligament fibroblast and minimal in gingival fibroblasts[49]. They are also found in liver, intestine and placenta. Measurement of total alkaline phosphatase activity is a useful indicator of bone formation when its source from bone is exceptionally high and from other sources are not increased and stable[51]. Hence a thorough medical history was undertaken for all the patients included in the study to rule out changes in enzyme activity due to other systemic reasons. Also periodontal examination was done to rule out periodontitis and throughout the study,

inflammation was kept under control by proper oral hygiene measures. **Randhir Kumar** showed that Alkaline Phosphatase levels are increased in the presence of gingival inflammation and periodontitis[15].

During childhood, the bone isoenzyme predominates[51]. **Perinetti *et al*** showed that Alkaline Phosphatase can be used as an indicator to assess pubertal growth spurt[82]. Hence to rule out the skeletal maturation levels of patients creating a bias in the study values, the age group for patient inclusion was narrowed to 18-21 years in this study.

Batra *et al* in their study found that in patients whom the leveling and aligning duration was increased, Alkaline phosphatase activity was also increased[21]. Hence the patient selection in this study was restricted to Angle's class I malocclusions with minimal or no crowding by using PAR index displacement scores, so that the enzyme activity due to major tooth movements prior to en-masse retraction was avoided.

Letitia *et al* found that the rate of bone remodeling was greater in mandible when compared to maxilla because of smaller volume of bone in maxilla[84]. Saliva does not show site specific changes to assess whether the enzyme activity in saliva was due to bone remodeling changes in maxilla or mandible. Hence in order to correlate the activity of Alkaline Phosphatase in GCF and saliva without any bias, only one arch, i.e., the maxillary arch was chosen for en-masse retraction.

Perinetti *et al* and **Batra *et al*** in their studies have shown that Alkaline Phosphatase activity was more in tension side than pressure side, since bone formation is more marked in tension site during orthodontic tooth movement[21,22]. Hence the site for collection of GCF, in this study, was chosen on the mesial side of right and left maxillary canine.

Alkaline Phosphatase is an enzyme that shows large inter-individual and also intra-individual variations due to various internal and external factors. The variations may be due to the genetic make-up of the enzyme system of an individual, circadian rhythm, stress levels, dietary habits, sleep cycle and so on[27]. In order to control the variations in enzyme levels due to circadian rhythm, all the samples were collected before the mid day meal and either before breakfast or 2 hours after breakfast to rule out the influence of diet. **Skidmore** states that there is no pre-determined value for this enzyme in saliva[27]. Hence the activity of the enzyme, before force application for en-masse retraction (T_0 values), were taken as the baseline value for comparison.

There are two main aims of this study. First, to understand the changes in Alkaline phosphatase levels during en-masse retraction as related to the time of treatment with a continuous force of 150gm and second, to find if the pattern of activity of this enzyme is same in saliva as in GCF, so that saliva can also be used as a medium to analyze Alkaline Phosphatase activity during orthodontic tooth movement.

Alkaline Phosphatase activity in GCF at different time intervals:

The results of the study show that there is an increase in Alkaline Phosphatase activity from baseline to 21st day. The increase from baseline to 1 hour after force application is less significant but a significant increase in Alkaline Phosphatase values is seen on the 3rdday, though this period is in the resorption phase of remodeling cycle. This may be due to bone formation of the modeling process on the tension side which is initiated in 40-48 hours[98]. A steep increase in alkaline phosphatase values is noted on the 7th day and the increase in values is continuing till the 14th day. This shows a strong correlation with the bone formation phase of remodeling cycle, which

lasts for 7-14 days. There is less significant increase seen on the 21st day which shows that the activity of the enzyme reduces after the formation phase.

The pattern of rise of enzyme studied from the incremental changes in the enzyme values at different time intervals also show that there is an increase in Alkaline Phosphatase activity and the peak activity of the enzyme is seen during the time interval from 3rd day to 7th day (T_2-T_3). There is a slight decline in the increase in enzyme from 7th to 14th day and a sharp fall in enzyme activity from 14th to 21st day, during which the activity of the enzyme returned to that seen during the activation phase of remodeling cycle (T_0-T_1). This may be due to the initiation of the next remodeling cycle, which is necessary for tooth movement to continue. **King and Keeling** studied bone remodeling in relation to appliance decay in rats and found that even with 93% appliance decay by day 16, a second wave of bone remodeling begins with abrupt inhibition of ongoing bone formation and initiation of second phase of bone resorption[43]. Since a continuous force is used in this study, the mechanical stimuli needed for the activation of subsequent remodeling cycle persists, which is reflected as the decline in rise of Alkaline Phosphatase activity after the 14th day. **King *et al*** studied alveolar bone turn over after removal of orthodontic appliances in rats and found that until 10 days after removal of the appliance there was delayed but sustained elevation in Alkaline Phosphatase levels but there was a relapse seen in tooth movement during this period. The enzyme pattern reversed in accordance with the direction of relapse only after 14 days. From this he says that, what is seen in tooth movement does not always reflect in the alveolar bone[45].

The results of this study concur with the findings of **Perinetti *et al*** who assessed Alkaline Phosphatase changes in GCF when distalizing molar with 250gm

continuous force for a period of 28 days[22]. **Insoft *et al*** in his longitudinal study in 3 patients found that there was peak in activity of Alkaline Phosphatase between weeks 1 and 3 followed by an increase in acid phosphatase activities between weeks 3 and 6. In 2 of the 3 patients he studied, the peak in activity was between weeks 1 and 2 followed by a sharp decline in activity at week 3[20]. This pattern is similar to the results of this study.

Contrary to this study, the study by **Batra *et al*** shows a peak in Alkaline Phosphatase activity on the 14th day followed by a steep fall in enzyme levels at 21st day[21]. Since the force values used in their study was 100gm for canine retraction, it could be the reason for the delay in peak activity due to the process of hyalinization and prolonged resorption. **Stephen Keeling *et al*** showed that force magnitudes can alter the timing of bone turnover events. In their study, three treatment groups of different force magnitudes (20gm, 40gm, 60gm) were studied in rat molars and they found with 40gm group peak Alkaline Phosphatase activity was seen on the 7th day but the enzyme activity in 20gm and 60gm group was less on the 7th day. In their study they suggest that at heavier force magnitudes, resorption phase is prolonged[19]. **Kohon *et al*** in their study have proved that when light forces are used there is no lag phase[57] as suggested by **Burstone[28]**. In this study, a continuous force of 150gm is used for en-masse retraction which seems to show optimal Alkaline Phosphatase changes during bone turnover.

In this study there is a less significant increase in enzyme levels in GCF between baseline and 1 hour, though the time interval is too short for osteoblastic activity to begin. The reason of this mild increase might be due to repeated sampling at the same site in a short duration, as said by **Michael Insoft *et al*** where the first

sample had significantly lower Alkaline Phosphatase activity than the subsequent samples[20].

Sarandeep Huja *et al* found in dogs that bone remodeling activity in anterior maxilla was higher than posterior maxilla[75]. In this study, the pattern of enzyme activity and growth rates are similar for both the sites of GCF, MX1 and MX2, which shows that there is no much difference in alveolar bone turnover changes between the right and left sides of anterior maxilla, provided the magnitude and type of force is maintained the same.

Alkaline Phosphatase activity in saliva at different time intervals:

In orthodontic literature very few studies have been done to analyze the biomarkers of tooth movement in saliva. **Andrea Marcaccini *et al*** studied the activity of Myeloperoxidase enzyme in GCF and whole saliva and found the pattern of changes in enzyme to be the same in GCF and saliva[16]. **Florez Moreno *et al*** confirmed that sRANKL and OPG can serve as analytes in a group of salivary biomarkers of orthodontic tooth movement[17].

This study was aimed to validate the role of saliva in assessing the changes in Alkaline Phosphatase activity during orthodontic tooth movement. The results of this study in saliva show that, similar to GCF, there is an increase in Alkaline Phosphatase levels from baseline to 21 days. There is no significant increase in values from baseline to 1 hour after force application but a gradual increase on the 3rd day followed by a steep rise on the 7th day is seen. The increase in Alkaline Phosphatase values continues till the 14th day. There is only a less significant increase in enzyme

levels on the 21st day. The pattern of rise of enzyme is also found to be similar to that seen in GCF.

To compare the Alkaline Phosphatase activity in GCF and saliva, Growth Rates were computed for each time interval in GCF and saliva separately. The maximum Growth Rates of Alkaline Phosphatase activity is seen from 3rd to 7th day in both GCF and saliva, which correlates with the peak in enzyme activity at the same time interval. Comparison of compounded growth rates in GCF and saliva shows that the growth rate of Alkaline Phosphatase is significantly less in saliva compared to GCF. **Navarro *et al*** in their study compared myeloperoxidase activity in GCF and saliva during orthodontic tooth movement. They concluded that GCF provides a more accurate picture of the enzyme activity than saliva as the source of GCF is closely related to the periodontal ligament and alveolar bone. They state that, though the contents of GCF are poured into the saliva, the main source of saliva is the salivary glands and they better represent the buccal environment than the alveolar bone changes[90].

The lesser growth rate of Alkaline Phosphatase in saliva might also be explained by the inhibitory effects of certain metal ions on the enzyme activity. **Robert Rej *et al*** found that metal ions of cobalt, nickel, cadmium, chromium, aluminium, manganese, iron and tin have an inhibitory effect on Alkaline Phosphatase activity[33]. **Mikulewicz *et al*** in an in-vitro study done for 28 days found that there is release of metal ions from orthodontic appliances into saliva. The release of ions is in the order of Si>Cu>Ni>Cr>Mo>Mn>Cd[91].

The Alkaline Phosphatase values obtained after assay for each patient was higher in saliva compared to GCF. This was due to the lesser quantity of GCF (1

microliter diluted to 20 microliters) that reacts with 200 microliters of the substrate (p-nitrophenylphosphate) to release p-nitrophenol, in contrast to 20 microliters of saliva reacting with 200 microliters of the substrate.

A major drawback in using saliva as a diagnostic medium to assess orthodontic tooth movement is that it does not show site specific changes and it is subject to the influence of various external factors such as diet. But the ready availability of saliva in sufficient quantities, ease of collection, time saving and money saving methods of sampling, ease of storage and assay, will definitely outweigh its drawbacks.

From this study it can be known that, although the growth rate of Alkaline Phosphatase activity in saliva is slightly less compared to GCF, still the pattern of rise of enzyme in saliva from baseline throughout the study period is similar to the enzyme activity in GCF (Graph: 2). This study concur with the findings of **Florez Moreno *et al*[17],** that unstimulated whole saliva can be an easy alternative to GCF when only phase specific changes linked to bone turnover during orthodontic tooth movement needs to be assessed but when accurate, site specific changes in bone turnover needs to be assessed then GCF seems to be the better option. Nevertheless, saliva could serve as a cost-effective diagnostic tool in daily clinical practice for screening of treatment mechanics and predict bone turnover in patients with systemic bone diseases and on chronic medications affecting bone metabolism[52].

SUMMARY & CONCLUSION

An in-vivo study was conducted in ten patients to compare the changes in Alkaline Phosphatase levels in Gingival Crevicular Fluid and saliva during en-masse retraction in the maxillary arch with a continuous force of 150 grams and also to check the validity of using saliva as a diagnostic tool to detect Alkaline Phosphatase levels during orthodontic tooth movement.

Saliva samples and GCF samples from the gingival sulcus in relation to mesio-labial line angle of right and left maxillary canines were collected and quantitatively estimated for Alkaline Phosphatase at baseline, 1 hour, 3 days, 7 days, 14 days and 21 days after force application.

The following inferences can be drawn from the study:

1. There is a definitive pattern of increase in Alkaline Phosphatase activity in both the sites of GCF and saliva from baseline to 21 days with a peak in activity on the 7^{th} day.
2. The pattern of rise of enzyme is the same in both GCF and saliva.
3. Growth rate of Alkaline Phosphatase from baseline to 21 days was less in saliva compared to GCF.

From this study it is concluded that Alkaline Phosphatase reflects changes in alveolar bone remodeling during en-masse retraction. This study also highlights that saliva can be used as an alternative diagnostic medium, which offers an easy, cost effective and time saving method to assess Alkaline Phosphatase activity during orthodontic tooth movement keeping in mind that it does not provide site specific changes in bone turnover. When accurate site specific changes needs to be assessed GCF still remains the viable option.

The current trend in orthodontics is shifting towards finding various methods to accelerate tooth movement at cellular and biochemical levels that alter the metabolism of alveolar bone. In such a scenario, the use of chair side diagnostic aids enables the clinician to find out the efficacy of new methods to accelerate tooth movement. Alkaline Phosphatase is already being routinely used as a regular diagnostic biomarker, in medicine and other fields of dentistry. Hence it can also be used as a chair side biomarker to assess bone changes during orthodontic tooth movement while its evaluation is simplified by using saliva as the medium for its detection. Further human studies, on a long term basis is necessary to reconfirm the validity of this study.

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